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(21) International Application Number: PCT/US92/02426 (22) International Filing Date: 20 March 1992 (20.03.92) (30) Priority data: 673,791 22 March 1991 (22.03.91) US (71)(72) Applicants and Inventors: GUERRANT, Richard, L. [US/US]; 2507 Northfield Road, Charlottesville, VA 22901 (US). REIN, Michael, F. [US/US]; 109 Sturbridge Road, Charlottesville, VA 22901 (US). BARRETT, Leah, J. [US/US]; Route 4, Box 180C, Charlottesville, VA 22901 (US). ARAUJO, Valter [US/US]; 2504 B Stadium Road, Charlottesville, VA 22901 (US).	(74) Agent: PARKER, Sheldon, H.; Parker & Destefano, 250 West Main Street, Suite 100, Charlottesville, VA 22901 (US). (81) Designated States: AT (European patent), AU, BE (European patent), BF (OAPI patent), BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), ML (OAPI patent), MR (OAPI patent), NL (European patent), SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: IN VITRO TEST FOR LEUKOCYTES IN BODY FLUIDS (57) Abstract An in vitro test for determining the presence of leukocytes in a body fluid or tissue sample which is sensitive to the numbers of leukocytes typically found in inflammatory processes, such as diarrheal specimens, by testing the fecal sample with an assay utilizing an antibody for lactoferrin. Additionally, the test can be used to detect low white blood cell counts as low as about 200 WBCs/ul.		

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IN VITRO TEST FOR LEUKOCYTES IN BODY FLUIDS

CROSS-REFERENCE TO RELATED PATENT APPLICATION

This application is a continuation-in-part of copending United States patent application serial number 442,309, filed 11-28-89, for New In vitro Test for Fecal Leukocyte, the subject matter of which is incorporated herein as though recited in full.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to diagnosis of inflammatory processes by examination of body fluids or tissues for leukocytes or white blood cells (WBC).

2. Prior Art

A need exists for a simple, reliable in vitro test for the diagnoses of inflammatory process, such as fecal leukocytes (even after they are morphologically disrupted), to indicate that a subset of patients with the common problem of diarrhea that is inflammatory and thus requires specific, more costly diagnostic and therapeutic attention.

Diarrheal illnesses are extremely common (causing 2 to 12 or more illnesses per person per year) throughout the world, and often pose diagnostic and therapeutic questions for the physician. Fortunately, important diagnostic clues can be obtained by considering whether the diarrhea is a noninflammatory process arising typically from the upper small bowel, or whether it is an inflammatory diarrhea arising from an invasive process in the ileum or colon. Although the majority of cases are noninflammatory and will often respond to simple oral rehydration therapy, it is im-

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1 portant to distinguish the invasive, inflammatory diarrheas,
2 which are usually caused by Shigella, Salmonella, Campylobacter
3 or Clostridium bacteria, that may be, more severe and should be
4 the focus of more expensive culturing for these invasive
5 pathogens, The invasive, inflammatory diarrheas may also require
6 specific antibiotic treatment. A particularly helpful diagnostic
7 clue to distinguishing inflammatory from noninflammatory diar-
8 rheas has been the examination for large numbers of leukocytes
9 (white blood cells or "pus cells") in the diarrheal fecal
10 specimens themselves. However, this requires that the physician
11 or promptly examine mucus from a cup fecal specimen under a micro-
12 scope, stained for clearly distinguishable leukocytes in the fe-
13 cal debris. This requires the immediate availability of a skilled
14 person with a microscope to stain and examine fresh fecal
15 specimens in the clinic or emergency area where the patient is
16 seen. Despite extensive efforts, this is difficult to accomplish,
17 especially with this extremely common problem in a busy clinic
18 setting.

19 There are many potential markers for leukocytes in the
20 primary and secondary granules. Leukocyte esterase was explored
21 as a potential marker for fecal leukocytes, since an analogous
22 test exists for leukocytes in urine. However, it was discovered
23 that leukocyte esterase was non-specifically positive for all
24 stool samples, both those with and without leukocytes.

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SUMMARY OF THE INVENTION

2 The diagnosis of inflammatory processes is best made by examina-
3 tion of body fluids, such as sputum, vaginal or cervical secre-
4 tions or tissue biopsies, for leukocytes. While this traditional
5 method requires a skilled microscopist examining stained material
6 on slides using a microscope, the WBCs may be destroyed by
7 pathogenic microorganisms or toxins or on transport or storage,
8 or absorbed onto swabs, or may be present in some areas, but not
9 other areas of the specimen. Further, the test is highly relevant
10 to the detection to dangerously low white blood cell counts, par-
11 ticularly neutropenia. Such low white blood cell counts severely
12 limits the less costly widespread use of emerging new drugs such
13 as clozapine (Clozaril) for schizophrenia or AZT for AIDS.

14 A simple, in vitro test for a leukocyte marker was developed
15 that is sensitive to the numbers of fecal leukocytes typically
16 found in inflammatory diarrheal specimens and that can be quickly
17 and easily done with a minimum of training, either in the clinic
18 later (after transportation or storage) in the laboratory.
19 Surprisingly, the test has been found to provide a simple sensi-
20 tive screen for as few as 200 WBCs/ul.

21 The marker found most specific for leukocytes in fecal
22 specimens is based on lactoferrin, an iron-binding glycoprotein
23 found concentrated in secondary granules in leukocytes
24 (Hetherington et al. "An Enzyme-Linked Immunoassay (ELISA) for
25 Measurement of Lactoferrin", J IMMUN METH, 65:183-190 (U.S.A.)
26 1983). Standard immunoassay methods of radial immunodiffusion,
27 latex agglutination and enzyme-linked immunosorbent assays

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1 (ELISA) that employ antibodies against lactoferrin have proved
2 successful in detecting specimens. However, the latex agglutina-
3 tion method seems the simplest and most applicable in vitro test.

4 These and further and other objects and features of the in-
5 vention are apparent in the disclosure, which includes the above
6 and ongoing written specification, including the claims and the
7 drawings.

8 BRIEF DESCRIPTION OR THE DRAWINGS

9 Fig. 1 graphically illustrates results of 22 fecal specimens
10 tested for C. difficile cytotoxin tested with the leukocyte lac-
11 toferrin latex agglutination assay showing that with increasing
12 cytotoxin titer, an increasing percent (to 100%) of lactoferrin,
13 evidence of an inflammatory process.

14 Fig. 2 graphically illustrates the increasing titer of
15 cytotoxin which is associated with increasing titers of lactofer-
16 rin, but decreasing appearance of leukocytes, which demonstrates
17 the destruction of leukocytes by the cytotoxin analogous to that
18 seen in vitro

19 DETAILED DESCRIPTION OF THE INVENTION

20 This in vitro test for a leukocyte marker must be sensitive
21 to the numbers of fecal leukocytes typically found in inflam-
22 matory diarrheal specimens. Typically, at least 5,000 to 10,000
23 polymorphonuclear neutrophils per mm³ are found in inflammatory
24 diarrheal specimens.

25 (a) Radial Immunodiffusion Assay

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1 Studies were conducted using the commercially available LC-
2 Partigen radial immunodiffusion plates (Calbiochem) for lactofer-
3 rin. The leukocytes were obtained from fresh, whole blood
4 processed with a neutrophil isolation kit. The fecal suspensions
5 with and without leukocytes were initially mixed with an equal
6 amount of 0.1% Triton-X to lyse the leukocytes (an addition that
7 was subsequently found to be unnecessary). Forty microliters of
8 this suspension was placed in the wells and the plates were
9 checked daily for visible rings around the wells. Within three
10 days, measurable rings were visible around the wells which had
11 stool samples with fecal leukocytes added and were absent in
12 samples without fecal leukocytes. The ring size with fecal
13 leukocytes at 10,000/mm³ was 9.05 mm. The ring size with fecal
14 samples that included 7,700 leukocytes/mm³ was 7.05 mm and no
15 ring was seen in fecal samples with no leukocytes. Similar
16 results were obtained after 10 days' refrigeration of the fecal-
17 leukocyte suspension as well.

18 Because the Calbiochem LC-Partigen kits became unavailable,
19 continuing work necessitated preparation of plates with purchased
20 anti-lactoferrin antibody. The preparation of plates for radial
21 immunodiffusion assay was made by adding 6 ml of 1% agarose con-
22 taining 1:1000 rabbit anti-lactoferrin antibody (30 ul per 6 ml
23 agarose per standard 2 x 3" slide). The rabbit anti-human lac-
24 toferrin was obtained from Sigma Company, St., Louis, Missouri
25 (Catalog # L-3262). The agarose was prepared using 1% low gel tem-
26 perature agarose (Sea Plaque from FM Corporation), 3%
27 polyethylene glycol-6000 from Fisher Scientific and 100 ml phos-

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1 phate buffered saline. When the agar covered slides were ready
2 for use, 2 mm wells were punched that each readily hold 5
3 microliters of antigen preparation each.

4 Using these methods for radial immunodiffusion, dose
5 response curves with purified lactoferrin (Sigma Chemical Com-
6 pany, St, Louis, Missouri) showed it was detectable at 0.02- 0.03
7 mg/ml (ug/mm³) with a ring size of 4.1 - 4.3 mm respectively.
8 Repeated studies with human PMN's (polymorphonuclear neutrophils)
9 revealed optimal sensitivity of approximately 2.08×10^3 MN/mm³,
10 giving a zone diameter of 4.7 mm. This corresponds to a calcu-
11 lated lactoferrin concentration of 0.0104 ug/ul based on the ap-
12 proximate concentration of 1 ng lactoferrin per 200 PMN's
13 (Hetherington et al. "An Enzyme-Linked Immunoassay (ELISA) for
14 Measurement of Lactoferrin", J IMMUN METH, 65:183-190 (U.S.A.)
15 1983). This result using 0.3% cetyl trimethyl ammonium bromide
16 (CTAB, a detergent used to lyse neutrophils for their release of
17 lactoferrin) was slightly better than that seen in the absence of
18 CTAB, and was in the same general range of radial immunodiffusion
19 assay results with purified lactoferrin noted above. Thus the sen-
20 sitivity of radial immunodiffusion appeared to be approximately
21 2000 PMN's/mm³, a number far lower than the expected concentra-
22 tion of leukocytes (PMN's) in inflammatory fecal specimens, judg-
23 ing by microscopy with numerous leukocytes per high power field.
24 Natural inflammatory diarrheal specimens revealed two patients
25 with documented Salmonella gastroenteritis showing distinct rings
26 ranging from 4 to 13 mm in size, as well as two patients with C.
27 difficile cytotoxin giving rings of 7.0 to 8.4 mm. In addition,

1 the normal stool specimen was repeatedly negative on three dif-
2 ferent occasions. CTAB and Triton detergents did not seem to add
3 any sensitivity to naturally inflammatory fecal specimens.

4 (b) Latex Agglutination Assay

5 For studies using latex agglutination, Bacto-latex 0.8I
6 beads (Code 3102, Difco Laboratories, Detroit, Michigan) were
7 coated with rabbit anti-human lactoferrin (Sigma Chemical Company
8 Product #L-3262, St. Louis, Missouri) as follows: 2.5 ml of beads
9 were centrifuged at 3000 rpm for 30 minutes, washed with 5 ml
10 glycine buffer (7.3 g glycine, 10 g NaCl, in 1 liter distilled
11 water adjusted to pH 8.2 - 8.3) and then resuspended in 5 ml of
12 glycine buffer to provide an approximate 1% suspension of beads.
13 To this latex bead suspension was added 0.35 ml rabbit antilac-
14 toferrin antibody and the mixture was incubated at 37 C for 1
15 hour, after which the antibody-coated beads were spun and
16 resuspended in 5 ml buffer to which 0.005 g azide (0.1%) and 0.05
17 g bovine serum albumin (1%) were added and the coated bead suspen-
18 sion was stored at 4 C until used. Studies with titrations or
19 purified Lactoferrin revealed readily apparent agglutination of
20 these latex beads with 0.004-0.0016 mg/ml lactoferrin, at least
21 one log more sensitive than the radial immunodiffusion (RID) as-
22 say mentioned above. This level of greater sensitivity of latex
23 agglutination was also seen with fecal-hypaque separated human
24 PMN's as well with a 1:100 dilution being positive when RID
25 detected only a 1:8 dilution. In addition, leukocytes added to
26 stools as well as the Salmonella and 4 C.difficile cases were
27 positive in the latex agglutination assay. Furthermore, three ad-

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1 ditional control specimens tested on 7 different occasions were
2 all negative. Importantly, these immunoassay results remained
3 clearly positive even after *C. difficile* cytotoxin totally
4 destroyed the MN morphology over 24 hours in refrigerated
5 specimens.

6 22 fecal specimens were tested with the leukocyte lactofer-
7 rin latex agglutination assay for *C. difficile* cytotoxin with the
8 results shown in figure 1. These results show that with increas-
9 ing cytotoxin titers, an increasing percent (to 100%) have Lac-
10 toferrin evidence of an inflammatory process. Remarkably, as
11 shown in Figure 2, the increasing titer of cytotoxin is as-
12 sociated with increasing titers of lactoferrin but decreasing ap-
13 pearance of leukocytes by microscopy, demonstrating the destruc-
14 tion of leukocytes by the cytotoxin analogous to that seen in
15 vitro.

16 Further data from children with diarrhea in the northeast of
17 Brazil have shown that specimens from 16 of 17 children with 1-5
18 or more fecal leukocytes per high power field on microscopy with
19 methylene blue stain had lactoferrin latex agglutination titers
20 of $\geq 1:50$. In contrast, only 3 of 12 methylene blue stained
21 specimens with less than 1 leukocyte per high power field had lac-
22 toferrin titers of $\geq 1:50$. Furthermore, despite occasional posi-
23 tives at lower titers, none of 7 specimens from normal control
24 children had lactoferrin titers of $\geq 1:50$.

25 (c) Enzyme-Linked Immunosorbent Assay (ELISA)

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1 Studies with the development of an ELISA for lactoferrin sug-
2 gest that it may be even more sensitive than the RID or latex ag-
3 glutination assays. However, as noted above, the need for in-
4 creased sensitivity may be unnecessary or even inappropriate. For
5 the ELISA testing, wells were coated with varying concentrations
6 of lactoferrin in a sodium bicarbonate buffer at room temperature
7 for 2-3 hours or overnight at 4 C. Wells and microtiter plates
8 were then washed 3 times with PBS-tween and 1% BSA was added to
9 fill the wells for 30-60 minutes to block nonspecific sites, fol-
10 lowed by washing 3 times with PBS-tween. Thereafter 50 ul of rab-
11 bit anti-human lactoferrin antibody (at 1:250 dilution, probably
12 optimal with 1:100 and 1:500 also being effective) was added to
13 each well, followed by 40 minutes incubation at
14 37 C (or 2 hours at room temperature), followed by washing 4
15 times in PBS-tween. Then 50 ul of goat anti-rabbit IgG (Rockland
16 Laboratories, Gilbertsville, Pennsylvania) with peroxidase con-
17 jugation (at 1:1000 dilution) was added for 40 minutes at 37 C
18 (or 2 hours at room temperature), followed by washing 5 times in
19 PBS-tween. Thereafter, 200 ul of activated peroxidase substrate
20 was added, followed by 45 minutes incubation at room temperature
21 in the dark, after which this was read both visually and
22 spectrophotometrically. The apparent sensitivity was 0,001 ug/uL
23 or less lactoferrin, with conjugate dilution of 1:1000 and
24 primary rabbit antibody dilutions of 1:250, probably representing
25 the optimal conditions for assay. The ELISA technology could
26 also be employed in detection of leukocytes, possibly using a
27 dipstick technology.

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1 While the invention has been described with reference to
2 specific embodiments, modifications and variations of the inven-
3 tion may be constructed without departing from the scope of the
4 invention, which is described in the following claims.

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1 What is claimed is:

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3 1. A method for the diagnosis of inflammatory processes, by
4 the examination of body fluids or tissues for leukocytes, compris-
5 ing the steps:

6 providing a body fluids or tissue sample containing
7 leukocytes;

8 testing said sample with an assay utilizing an antibody that
9 is sensitive to the number of leukocytes, using a specific
10 leukocyte marker; and

11 observing said sample for the presence of the specific
12 leukocyte marker.

13

14 2. The method of claim 1, wherein the specific leukocyte
15 marker is lactoferrin.

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17 3. The method of claim 1, wherein a radioimmunodiffusion
18 assay is utilized.

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20 4. The method of claim 1, wherein a latex agglutination as-
21 say is utilized.

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23 5. The method of claim 1, wherein a enzyme-linked immunosor-
24 bent assay is utilized.

25

26 6. The method of claim 1, wherein the body fluid or tissue
27 is sputum, or a vaginal or cervical secretion.

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2 7. The method of claim 6, wherein said diagnosis is for
3 purulent pneumonitis, vaginitis or cervicites.

4

5 8. A method for the detection of low white blood cell
6 counts by the examination of body fluids or tissues for
7 leukocytes, comprising the steps of:

8 providing a body fluid or tissue sample containing
9 leukocytes;

10 testing said sample with an assay utilizing an antibody that
11 is sensitive to the number of leukocytes, using a specific
12 leukocyte marker; and

13 observing said sample for the presence of the specific
14 leukocyte marker.

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16 9. The method of claim 8, wherein said detection is sensi-
17 tive to as few as about 200 white blood cells/ul.

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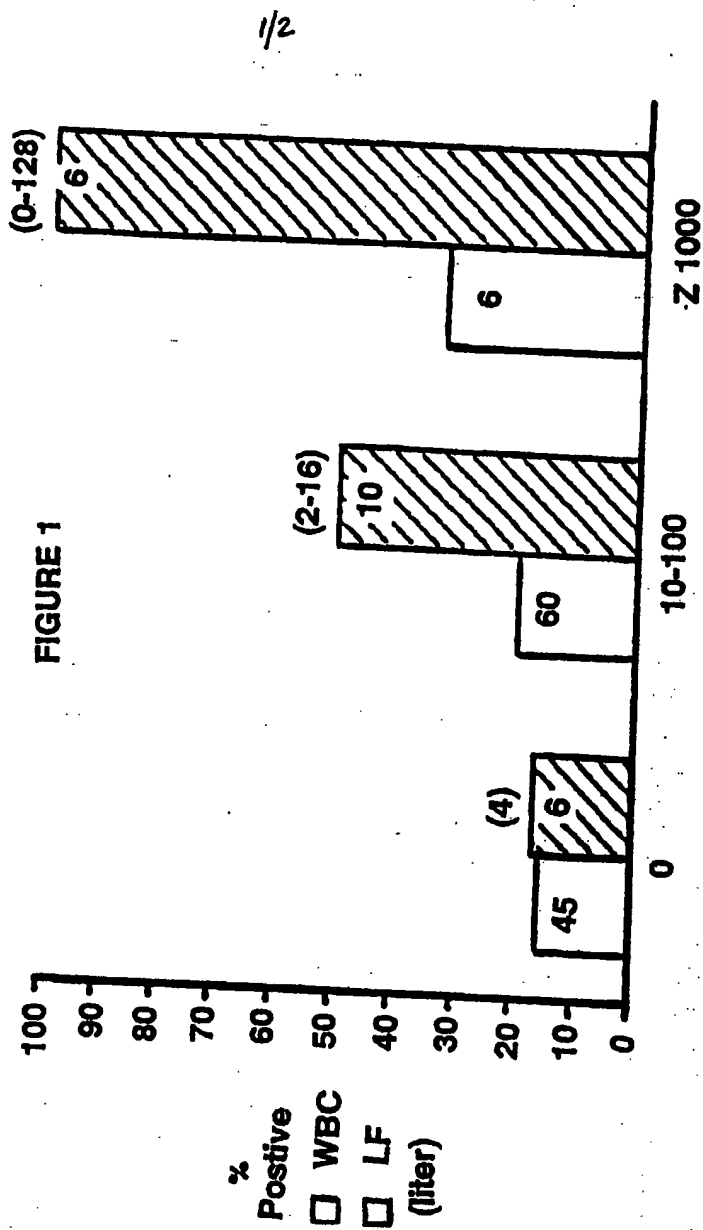
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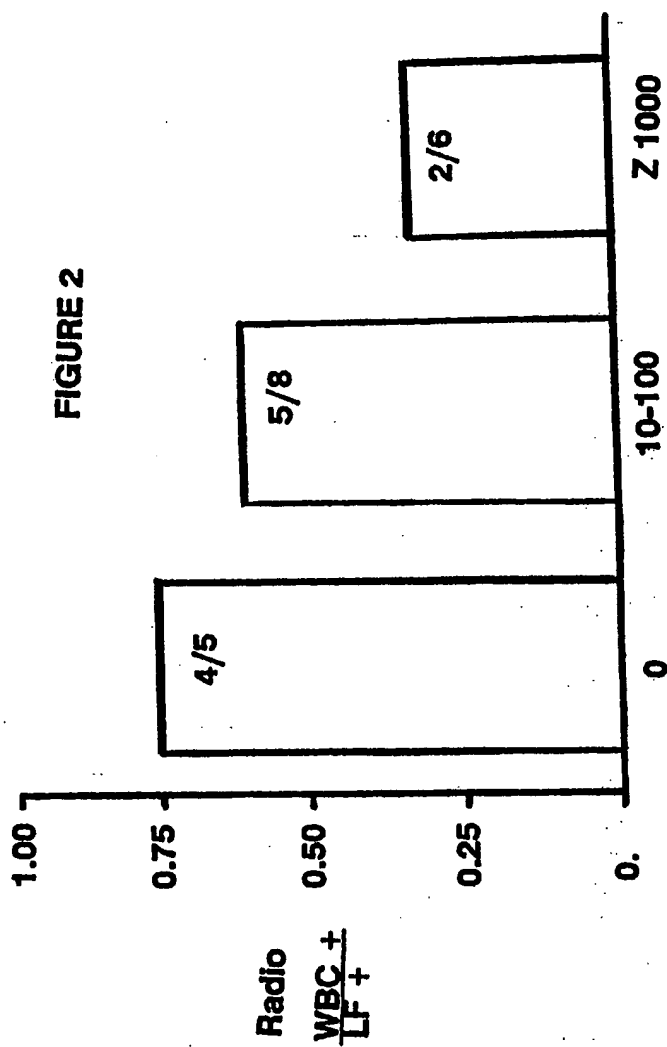
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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02426

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): G01N 33/545, 33/546		
US CL : 435/7.92, 7.94; 436/514, 533, 534		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/7.92, 7.24; 436/514, 533, 534	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
Lactoferrin		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁹	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁰
Y	Journal of Immunological Methods, Volume 65, issued 1983, S. Hetherington et al., "An Enzyme-Linked Immunoassay (ELISA) for Measurement of Lactoferrin", see pages 188-190, last paragraph.	1, 2, 5-9
Y	American Journal of Medicine, Volume 78, issued June 1985, R. Guerrant et al., "Evaluation and Diagnosis of Acute Infectious Diarrhea, pages 90-98, see abstract.	1-9
Y	Am. J. Trop. Med. Hyg., Volume 28, Number 6, issued 1979, O. Korzenowski et al., "Value of Examination for Fecal Leukocytes in the Early Diagnosis of Shigellosis", pages 1031-1035, see abstract.	1-9
Y	Infection and Immunity, Volume 6, issued November 1972, M. Leffell et al., "Association of Lactoferrin with Lysozyme in Granules of Human Polymorphonuclear Leukocytes", pages 760-765, see Figure 3.	1-3, 6-9
Y	US, A, 4,184,849 (Cambiasso et al.) 22 January 1988, see column 1, lines 1-36 and column 2, line 67 - column 3, line 5.	1, 2, 4, 6-9
<p>¹⁸ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ²
30 JUNE 1992		20 JUL 1992
International Searching Authority ¹		Signature of Authorized Officer ²⁰
ISA/US		MARY E. CEPERLEY ROZ

Form PCT/ISA/210 (second sheet)(May 1986) B

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
X, Y	E, US, A, 5,124,252 (Guerrant et al.), 23 June 1992, see claim 1.	1-9
V. <input type="checkbox"/> OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹		
<p>1. <input type="checkbox"/> Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:</p> <p>2. <input type="checkbox"/> Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:</p> <p>3. <input type="checkbox"/> Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).</p>		
VI. <input type="checkbox"/> OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²		
<p>This International Searching Authority found multiple inventions in this international application as follows:</p> <p>1. <input type="checkbox"/> As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.</p> <p>2. <input type="checkbox"/> As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:</p> <p>3. <input type="checkbox"/> No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:</p> <p>4. <input type="checkbox"/> As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.</p> <p>Remark on protest</p> <p><input type="checkbox"/> The additional search fees were accompanied by applicant's protest.</p> <p><input type="checkbox"/> No protest accompanied the payment of additional search fees.</p>		